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14. ABSTRACT The main objective of the study is the assessment of microsatellite markers with loss of heterozygosity (LOH) in serum, bone marrow (BM), and primary tumors. In addition the study involves the assessment of circulating tumor cells in blood if they correlate with serum circulating DNA. This is the final progress report of the study. The study was delayed due to logistics of the Office of Regulatory Compliance and Quality, U.S. Army Medical Research and Materiel Command and our Human Subjects IRB. The project has continued on and is now completed. We have successfully demonstrated LOH detection in breast cancer patients serum, bone marrow, and tumors. During the course of the study we have developed an optimal assay using capillary array electrophoresis. The assay is more sensitive than the original gel assay proposed. The study demonstrated LOH in serum was a significant prognostic factor.				
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Introduction

Initiation and progression of breast cancer involves the serial accumulation of a variety of genetic (DNA) alterations to the cell (1). One such event involves loss of heterozygosity (LOH) of DNA which has been shown to occur frequently in primary breast cancers and more so in metastatic disease (2). It has been suggested that these allelic imbalance (AI) are associated with tumor progression may involve potential tumor suppressor genes and/or metastasis regulator genes (3,4). Detection of these circulating tumor-associated DNA markers in the blood from cancer patients may serve as surrogate markers of subclinical disease progression and permit identification of high-risk patients early in their disease course (4-6). Furthermore DNA based genetic testing of serial blood samples offers an easily accessible route by which to evaluate ongoing genetic events which may have prognostic significance (7). This new approach provides for a minimally invasive method to monitor subclinical disease progression and offers a novel technique for potentially assessing response to therapeutic interventions in early stage breast cancer patients (8). We have successfully demonstrated the detection of circulating DNA microsatellites for LOH in the serum of breast cancer patients (9) and have shown its potential utility as a prognostic factor. During the grant period we have developed a methodology to allow for high throughput and multiplexing of DNA markers in order to more efficiently and accurately evaluate multiple samples. This high-throughput assay for LOH is performed by PCR and capillary array electrophoresis (CAE). During the grant period we have demonstrated the unique finding that free DNA can be detected in bone marrow aspirates and this DNA consists of microsatellites with LOH. The grant period was delayed due to logistic problems in the human IRB consent. However, once approved the aims of the proposed studies were carried out. This is the final report of the study.

Body

Aim Ia: Assessment of microsatellite markers for LOH in serum.

In this Aim, we assessed breast cancer patients' serum for LOH using a panel of multiple polymorphic microsatellite markers on multiple chromosomes. DNA was isolated from serum obtained pre-operatively from AJCC stage I-III patients and at the time of diagnosis from AJCC stage IV patients. Initial studies for LOH analysis were performed by gel electrophoresis using a fluorescent scanner and ClaritySC 3.0 software (Media Cybergenetics). We then converted the post-PCR fragment separation and detection to a capillary array electrophoresis (CAE) (Beckman CEQ 8000 XL) assay. We compared the assay sensitivity and specificity between the gel electrophoresis/fluorescent scanner and the CAE. The sensitivity and throughput capacity was much improved using the CAE. Samples previously assessed by gel electrophoresis were validated by the CAE. Samples are to be run in triplicate. Serum was collected from healthy female donors and assessed in a similar manner. This new approach provided high-throughput analysis with greater sensitivity and specificity. The specificity was up to 1-2 bp differences.

Overall, we obtained 146 patients: 122 AJCC stage 1-III and 24 AJCC stage IV patients. The serum was processed, DNA extracted, and quantified. For controls 50 healthy donor individuals, seven DCIS (ductal carcinoma in situ), and two patients with benign disease. At least one LOH marker was identified in serum samples from the 44 (30%) of the 146 patients. The most frequent marker detected was D16S421 occurring in 15 (10.3%) patients followed by D17S849 in 11 (7.5%) patients, D8S231 in 10 (6.8%) patients, D17S855 and D10S197 in 9 (6.2%) patients each, D14S62 in 8 (5.5%) patients, D14S51 in 6 (4.1%), and TP53 in 2 (1.4%) patients. None of the healthy donor volunteers, benign breast disease or DCIS had LOH. There was a statistically significant correlation of LOH presence and AJCC stage ($p < 0.001$). LOH was present in 7 (13%) of 54 AJCC stage I patients, 13 (26%) of 51 AJCC stage II patients, 8 (47%) of 17 stage III patients, and 16 (67%) of 24 stage IV patients. Patients were assessed in regards to standard known prognostic factors such as tumor type and size, ER, PR, grade, DNA ploidy, S-phase fraction, Ki67 index, HER2 receptor, p53 overexpression and lymph node involvement. There was a significant correlation to tumor size with LOH in serum ($p < 0.027$). Advanced tumor grade was also significantly associated with the presence of serum LOH occurring in 13 (34%) of 38 patients with grade 3 tumors as compared to 12 (15%) of 81 patients with grade 1 or 2 tumors ($p = 0.016$). There was a statistically significant correlation between the presence of serum LOH and the extent of lymph node involvement according to the pathology node status (pN) classification of the AJCC staging system. Eleven (14%) of 77 patients with no lymph node metastasis (pN0) demonstrated LOH in

serum whereas 9 (30%) of 30 patients with pN1 and 6 (46%) of 13 patients with pN2/pN3 disease demonstrated serum LOH ($p < 0.02$).

A logistic regression model was developed to examine the relation of AJCC stage, nodal involvement, tumor size and grade with the serum LOH for patients with AJCC stage I-III disease ($n=122$). A stepwise procedure was used for variable selection. Compared to patients without pathologic lymph node involvement (pN0), patients with pN1 disease had an estimated odds ratio = 2.44 (95% confidence interval = 0.85 to 6.98) and pN2/pN3 had an estimated odds ratio = 5.49 (95% CI = 1.53 to 19.69) for the presence of circulating tumor DNA for LOH in serum, respectively.

Aim Ib. Assessment of LOH markers in fluid of bone marrow (BM) aspirations.

For this Aim, because bone is such a common site of breast cancer recurrence, we sought to determine whether microsatellite markers associated with breast cancer could be detected in BM aspirates from patients with early stage breast cancer. Cell-free plasma from BM aspirates in 48 patients was collected intra-operatively from patients undergoing surgery for their primary breast cancer diagnosis (manuscript #3). This was a retrospective study of BM aspirates previously collected. Eight polymorphic microsatellite markers which correspond to regions that have been shown to demonstrate significant LOH suggesting sites of putative tumor suppressor and/or metastasis related genes were selected: D1S228 at 1p36; D8S321 at 8qter-8q24.13; D10S197 at 10p12; D14S51 at 14q32.1-14q32.2; D14S62 at 14q32; D16S421 at 16q22.1; D17S849 at 17pter-17qter and D17S855 at 17q (manuscript #5). Methods are being developed to optimize extraction of DNA from BM plasma. DNA was isolated from acellular fluid obtained from BM aspirations, purified and quantified. Paired lymphocytes were collected from each patient and DNA extracted to serve as normal control for each PCR reaction. Following PCR, fragments will be assessed using the CAE assay system. Cell free plasma from BM aspirates in 48 patients was assessed: LOH was detected in 11(23%) of patients' BM. In 24 patients paired blood serum samples were available for analysis and showed more frequent detection. The detection frequency of markers was D14S62 (12%), D14S51 (8%), D1S228 (8%), D8S321 (5%), D10S197 (4%), D17S855 (3%), D17S849/D16S321 (0). There was an increased association between the presence of LOH in the BM and advanced disease stage: AJCC stage I (19%), AJCC stage II (31%), and AJCC stage III (50%). The study successfully demonstrated the presence of LOH markers in early stage BM. This is an important and significant finding since breast cancer has a high propensity to metastasize to the BM early in the course of tumor progression.

Aim Ic. Correlation of LOH microsatellite markers in paired bone marrow and primary tumors.

To determine whether a correlation existed between LOH detected in early stage breast cancer patients' bone marrow and primary tumors, we assessed match-paired specimens with identical microsatellite markers. DNA was isolated from paired primary tumor and bone marrow specimens and analyzed using 8 microsatellite markers (D8S321, D10S197, D14S51, D14S62, D16S431, T53, D17S849 and D17S855). There was an association of primary tumor size and LOH. Lobular carcinomas were more likely associated with increased LOH in BM aspirates than infiltrating ductal carcinoma tumors ($p < 0.006$). The concordance of primary tumors LOH with detection of BM LOH was >90%.

Aim IIa. Assessment of circulating tumor cells in blood by qualitative RT-PCR and correlation to LOH in serum

In this study, we will assess blood from breast cancer patients diagnosed with breast cancer. RNA was extracted from cells in the blood and will be analyzed using a quantitative Realtime RT-PCR assay. The previously developed assay using ultrasensitive electrochemiluminescence detection solution phase technology (IGEN) with specific probes for the presence of four tumor markers { β -HCG (human chorionic gonadotropin), c-MET (HGF receptor), GalNAc (glycosyltransferase), STC-1 (stanniocalcin-1) and MAGE-3 (melanoma associated antigen-3)} were converted to the quantitative Realtime RT-PCR assay. Blood was collected and being assessed with matched-paired serum samples for LOH. These studies are ongoing and will be completed in several months. The studies were delayed due to establishing and optimizing the marker assays for quantitative realtime RT-PCR. These studies will be completed by separate funding.

Aim IIb. Assessment of circulating tumor cells in blood by quantitative RT-PCR

Currently, we are developing and optimizing markers used in IIa for quantitative RealTime RT-PCR (qRT). Primers, probes and standards have been developed and tested on normal and patients' blood. The assays for individual markers are being evaluated on controls for specificity and sensitivity. The optimized assays will be used for assessment of blood from patients. During our studies we developed a new RT-PCR marker referred to as HMW (high molecular weight) marker was originally discovered in melanoma but has now been detected in breast cancer cells at approximately 30-40% depending on the stage of disease. This new marker is being pursued for specificity and sensitivity currently in addition to the other markers. Circulating tumor cells are detected in breast cancer patients relative to advancing AJCC stage. However, the utility is not known until adequate follow up time is carried out particularly the stage I and II patients.

Aim IIc. Assessment of isolated tumor cells in blood

We have been investigating apoptotic cells in culture and tissue sections by immunostaining. The objective was to develop an optimized assay that can be informative and reliable. We found that single cells are difficult to assess for apoptosis whereby, clumps of cells were easier to assess. Currently we are developing techniques to improve isolation of tumor cells from blood. Different types of magnetic beads and antibodies are being tested to get optimal cell isolation. The study to date was not successful. The major problem is the lack of specificity of antibodies to breast tumor cells. This has hampered the study.

Aim IIIa. Neoadjuvant treatment analysis

To date we have collected, processed and isolated DNA from patients' from patients receiving neoadjuvant treatment. However, we have not yet run these samples. In these patients we will be assessing LOH markers in serum and circulating tumor cells. These will be run in the near future as follow up is not completed yet.

Key Research Accomplishments

- Conversion of the gel electrophoresis/fluorescent scanner assay for assessment of microsatellite to capillary array electrophoresis (CAE).
- Conversion of IGEN electrochemiluminescence RT-PCR detection assay to a quantitative Realtime RT-PCR assay.
- Establishment that microsatellite with loss of heterozygosity (LOH) can be detected as free DNA in breast cancer patients.
- Circulating DNA as LOH of microsatellites can be detected in bone marrow.
- Correlation of LOH markers in breast cancer patients' serum as a significant prognostic factor.

Reportable Outcomes

A. Manuscripts

1. Taback, B., Shinozaki, M., Fujimoto, A., Nakayama, T., Hoon, D.S.B. Circulating tumor-related DNA as prognostic markers. In: Cancer Diagnostics: Current and Future Trends. R. Nakamura, W. Grody, J. Wu, R. Nagle (eds) Humana Press Inc, Chapter 19, pp 395-428, 2004.
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B. Abstracts

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Conclusions

The study demonstrated that microsatellite markers with LOH can be detected as circulating DNA in serum and in bone marrow aspirates. The first most significant discovery was the presence of these DNA markers in serum with increasing levels as disease stage advanced. The second most significant finding is that these DNA markers were significant prognostic factors. The third important finding is the detection of DNA markers in early stage bone marrow aspirates. The study accomplishments are quite significant and move the field of circulating DNA forward.

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